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(54) Measurement of somatic cells in milk

(57) In the detection of mastitis and measurement of hygienic quality of milk, somatic cells in a sample of the milk are treated to release ATP, the sample is mixed with firefly luciferin-luciferase reagent, and the light intensity emitted by the sample is measured with a photometer. A portable photometer is described for field measurement of somatic cells in milk or in other types of samples where luminescent measurements are done in field conditions.

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FIG. 1

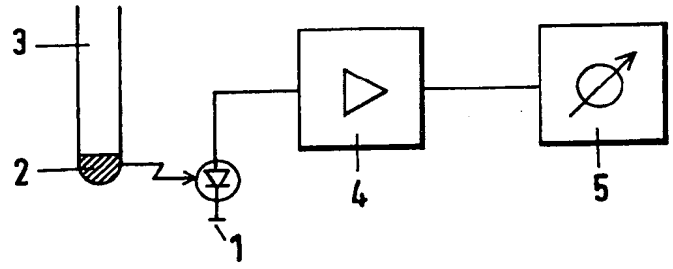
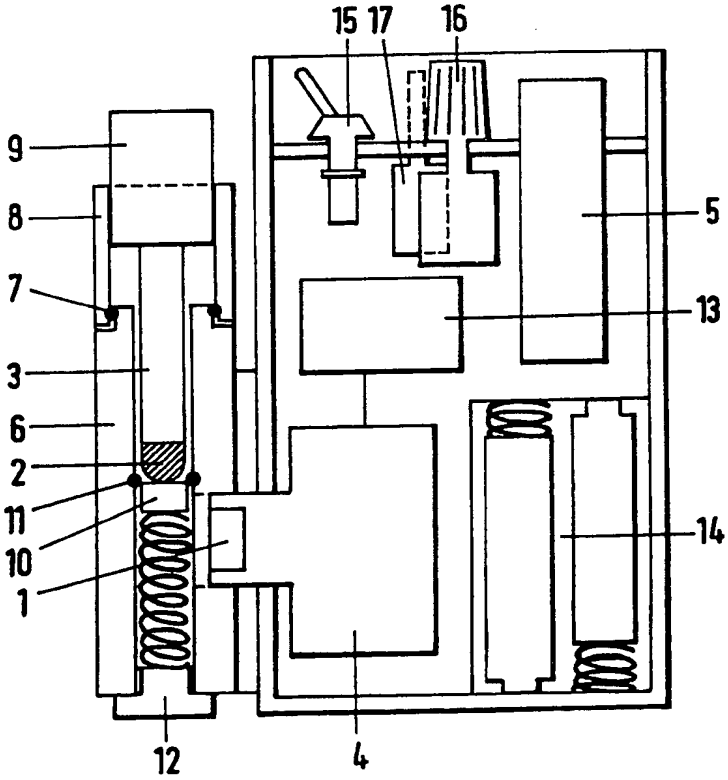


FIG. 2



SPECIFICATION

Measurement of somatic cells in milk

This invention relates to fast and accurate determination of somatic cells (leucocyte and epithelial cells) in milk by means of a simple assay of adenosine triphosphate from somatic cells by means of firefly bioluminescence.

In veterinary, animal husbandry and diary industry the detection of mastitis (baceterial infection of udder) in cows is of great importance for the wellbeing of cows themselves, minimizing the economical losses due to reduced milk production as well as protecting the human population against transmission of pathogenic bacteria from milk. Mastitis is detected by means of measuring the quantity of somatic cells. Healthy cows have a somatic cell count normally below 150.000 per ml with some variation due to the physiological state of the animal. In chronic sub-clinical mastitis somatic cells exceed 500.000 per mol and in acute mastitis somatic cell count may be as high as several millions per mol. Conventional methods of enumerating somatic cells in milk are based on:

1. direct microscopic counting,
2. precipitation of DNA (desoxyribonucleic acid) by chemicals, such as detergents in so called "California Mastitis Tests" (CMT),
3. Counting cells in an electronic particle counter, or counting the cells with a fluorescence counter..

All these methods have certain drawbacks: direct microscoping counting is slow and too laborious for large number of samples in today's large diaries; CMT and other precipitation methods are subjective and difficult to automate; electronic particle counters are expensive and subject to erraneous results due to the fat globules in milk, and the cell counters based on fluorescence are too expensive for small and medium size diaries and laboratories.

. It is an object of the present invention to provide a sample, fast accurate and economical method of determination of somatic cells in raw milk, which will easely lead to automation and is suitable both for field testing at farms and also measurements in the laboratory of dairy facilities.

Another object of the invention is the provision of an automatic apparatus by means of which the determination of the somatic cells in milk may be performed. The measurement is based on the well known firefly bioluminescence measurement of adenosine triphosphate (ATP) from cells (see U.S. Patent No. 3,745,090).

In the present invention the firefly bioluminescence method is applied to selective measurement of somatic cells in raw milk.

Measurement is made as follow, 10–100 μl, preferably 20 or 50 μl of raw milk (stored between 0-5°C, but no longer than 36 hours) is pipetted into a measuring vial. Hereto is added 10–500 μl, preferably 50 or 100 μl, of Nucleotide Releasing Reagent for Somatic Cells (NRS, a registered Trade Mark of Lumac System AG, Basel) (see British provisional patent application no. 22992/77) and shake. The vial is placed to a luminescence photometer and 10–100 μl, but preferably 100 μl of firefly luciferin-luciferase reagent in a buffer having pH between 7.0–8.2, but preferably 7.75 is dispensed and the light intensity, produced by the sample, is measured. The concentration of ATP or the number of somatic cells in then calculated by correlatin the sample reading to that of a known standard.

An alternative way of measurement is to add a 20–100 μl milk sample to a cuvette containing 100-500 μl of a mixture of luciferin-luciferase in buffer having pH from 7.0–8.2, but preferably 7.75, and 0.1% NRS reagent. After mixing the sample and reagent, mixture is ready for measurement.

According to the method of the invention ATP is selectively relased from somatic cells, which of course, is important because raw milk can contain large numbers of normal milk bacterial fauna, and so if ATP from bacteria is released, this will cause an error in the somatic cell counting by means of firefly bioluminescence.

In United States Patent No. 3,745,090 alternative extraction procedures for ATP are described but these interfere with the luciferin-luciferase reaction or are tedious and difficult to automate.

ATP results, obtained with the present method, correlate to the cell numbers counted with an electronic particle counter with a correlation coefficient of 0.93–0.97. Correlation to the CMT (California Mastitis Test) and fluorescence cell counter were also between 0.9–1.0 (Fig. 1).

Measurement of somatic cells with the present invention and a manually operated luminescence photometer takes 20–40 sec. per sample, thus the method has a high throughput. With an automatic instrument the rate of measurement can be as high as 200–500 samples per hour. A further advantage of the method is that it can be easily performed at the farm with a portable meter by the farmers themselves, drivers of the milk collecting tank trucks, veterinarians or other people concerned about the hygieny of milk or the health condition of the cows. The measurement is so easy that it can be performed and the results interpreted by anybody without training.

The apparatus to carry out the measurement can be used with a variaty of luminescent systems such as luminescent measurement of peroxide (H_2O_2) or superoxides O_2 with luminol (3– aminophtalhydrazide) of flavin mononucleotide (FMN) or nicotinamide adenine dinucleotide or its phoshate in re-

duced form (NADH or NADPH) with photobacterium bioluminescence, etc., bioluminescent assay, but preferably with the firefly luciferin-luciferase bioluminescent measurement of ATP, such as the measurement of the number of somatic cells in raw milk through the quantity of ATP in the samples, or quantitizing of other organism through the concentration of ATP in the samples.

As an example of the use of the apparatus the estimation of somatic cells in raw milk is described below, using specialized reagents that produce a long-lasting light signal that is easy to record with the present apparatus:

A firefly luciferin-luciferase reagent is prepared, including 0.005–0.02 molar Mg^{2+} salt in tris, glycylglycine, mops etc. biochemical buffer having pH 7.0–8.2, but preferably LUMIT a registered Trade Mark of LUMAC SYSTEMS AG in Hepes buffer containing 0.01 molar $MgSO_4$, 0.001 versane chelator and 0.1% NRS a registered Trade Mark of LUMAC SYSTEMS AG and whereafter 0.05–1.0 ml, but preferably 0.1–0.5 ml to a transparent cuvette is dispensed.

10–100 μl, but preferably 50 μl of raw milk is pipetted into the cuvette containing the luciferin-luciferase–NRS–buffer reagent and mixed for 10–15 seconds.

The cuvette is placed into the reaction chamber of the luminescent photometer and the cap is placed on the top of the reaction chamber.

The dark current at the diode is zeroed with the zeroing potentiometer; the cap is pressed down and the voltmeter reading after reaching the maximum value is recorded.

The reading is then converted to desired parameter by comparing the reading to that of a known standard, or the instrument is calibrated before sample measurement with a known standard to give the readings directly in the number of somatic cells per milliliter.

The present invention also provides a simple, inexpensive photometer suitable for measurement of luminescent reaction in field conditions, such as the measurement of somatic cells in milk. The photometer, however, may also be used for the measurement of the activity of activated sludge in sewage treatment plants, biomass of aquatic organisms, biomass of soil microbes etc. measurements based on the firefly bioluminescent measurement of ATP or other luminescent reactions lasting long enough (more than a few seconds) in order to make a visual reading.

The measurement of light produced by luminscent reactions such as the firefly luciferin-luciferase bioluminescent system for quantitizing ATP is performed by photometer or photon counters. Photometers apply the measurement of light intensity with a detector such as photmultiplier tube or photosensitive cell such as silicon or gallium-arsenite diode. These detectors convert the light energy to electrical current which is recorded by an analogue or digital voltmeter, a strip chart recorder or an oscilloscope. Photon counters use a photomultiplier as a detector and record the light intensity as high amplified (10^4–10^9 times) electric pulses produced by single photon striking the photosensitive cathode.

The photometer according to the invention is illustrated by way of example in the drawing in which:

Figure 1 shows a schematic diagram of the electrical circuitry of the protable luminscent meter; and

Figure 2 shows a schematic diagram of the mechanical components of the luminescent photometer for measurement of somatic cells in milk.

The instrument consists principally of a silicon photodiode, designated by 1 in Fig. 1 that converts the light intensity emitted by the sample 2 in a cuvette 3 to an electrical current amplified by the preamplifier 4 and recorded by an analogue voltmeter 5 as a continuous signal.

The construction of the portable luminescent photometer is illustrated in Fig. 2. The sample 2 is contained in a transparent cuvette 3 in the light-tight reaction chamber 6 which is made light-tight with an O-ring 7 and a cap 8 consisting of an inner plug 9 that is used to push down the cuvette 3 in such a way that the elevator 10 that makes a light-tight seal with an O-ring 11 when the camber is empty, is pushed down to lower the sample 2 in front of the silicon diode detector for the measurement of the light intensity of bioluminescence emitted by the sample in the presence of the firefly bioluminescent reagents. A threaded cap 12 seals the reaction chamber from the bottom. The electrical current coming from the diode 1 is amplified in the preamplifier 4 and the scaling circuitry 3 and displayed by an analogue voltmeter 5. Before the measurement the electrical operation current is turned on with the switch 15, the instrument is manually zeroed with a potentiometer 16 and the sample in a cuvette placed in the reaction chamber. After the cap 9 is pushed down, the voltmeter 5 will show the light intensity as signal (incident light intensity at the given time). Apparatus can be calibrated to give readings in number of cells or concentration of ATP or any other substrate utilized in the luminescent systems by measuring a known standard and adjusting the reading of the voltmeter to correspond the measured parameter by means of a adjustable amplifying potentiometer 17.

CLAIMS

1. A method whereby the somatic cell count in milk is measured by means of firefly luciferin-luciferase assay of adenosine triphosphate (ATP) in the sample.

2. A method according to claim 1 where

the milk sample is treated with a nonionic surface active agent to release ATP selectively from somatic cells.

3. A method according to claim 1 where the concentration of ATP is measured in a luminescent photometer after addition of firefly luciferin-luciferase reagent in a buffer having a pH value between 7.4 and 8.0 and a magnesium concentration of 0.005 — 0.02 moles per liter.

4. A method according to claim 1 where the concentration of ATP is converted to the number of somatic cells per milliliter by applying conversion factor determined by means of measurement of a known standard.

5. A method according to claim 1 where the number of somatic cells is measured in the field with a portable photometer instrument by means of ATP concentration in milk sample.

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